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I. LOW NICOTINE STUDY

A. Objective: To investigate the biochemistry of the nicotine biosynthetic pathway and use this information to generate tobacco plants with reduced nicotine levels.

B. Results: Nine batches of roots from Group 29 hydroponically grown Burley 21 tobacco plants have been processed through the 40-65% ammonium sulfate stage. These plants were harvested 3 days after topping. Root extracts were low in enzyme activity and in protein concentration. Specific activities (S.A.) ranged from 10.3-45.5 nmoles/mg protein (units) at 30°C for 30 min. These activities were similar to the activities obtained from roots of plants harvested in past summers. A study is currently in progress in the greenhouse to correlate the outside air temperature to temperatures in the greenhouse and the hydroponic solution (1).

Ammonium sulfate stage samples were pooled and fractionated on two 500 ml bed volume phenyl-Sepharose columns. The specific activity for the peak PMT samples of the first column was determined to be 132.5 units (a 7.5 fold difference in purification compared to the ammonium sulfate fraction). The second column is currently being processed (1).

Ammonium sulfate samples (PMT 242) were analyzed on the RF3 isoelectric focusing unit. Based on the results of these two analyses, no additional ammonium sulfate fractions will be examined on the RF3 unit (2).

Experiments have been conducted for the optimization of the liquid renaturation procedure. Inclusion of dialysis buffer/DTT washing prior to incubation with Extracti-Gel and putrescine appears to enhance renaturation. Renaturation was possible after the starting material was denatured by the addition of Seprasol and boiling after 2 and 5 minutes (3).

Analysis by HPLC of derivatized renatured material subjected to the enzyme assay confirmed the presence of two radioactive peaks, with one corresponding to NMP (3,6). Extracti-Gel does not appear to act as substrate with phenyl-Sepharose material (6). Tris buffer was determined to be the best buffer compared to Tricine, Bicine, EPPs, or sodium bicarbonate for the enzyme assay (4,6).

Work is in progress using HPLC to resolve the components which make up the 60 kD molecular weight region (5).

Two differentially expressed root specific tobacco cDNA clones were digested with EcoR1/XhoI to determine the size of the clone insert. The restriction digests were Southern blotted for further study (7). Several oligonucleotides were synthesized in-

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house for anti-sense studies. A relatively quick method to purify oligonucleotides in-house was developed. Several oligonucleotides were purified by this method and then used for cloning and Polymerase Chain Reaction (PCR) work. Restriction mapping of various tobacco root clones was conducted to yield materials for sense and antisense cloning technique development (8). A commercially available plant vector, pBI121 (Clonetech), was modified to facilitate anti-sense cloning of tobacco root cDNA insert. The glucuronidase gene (Gus) was cloned into a pBluescript vector. Studies are under way to confirm and characterize these constructs (9,10).

Burley 21 protoplasts were electroporated at varying voltages in the presence of pHIL101 plasmid which confers kanamycin resistance and β-glucuronidase (GUS) activity. In addition DNA prepared from a root-specific clone (PR17) was also electroporated into the cells. These cells are being cultured prior to assay for Gus and/or kanamycin selection. In a preliminary experiment Agrobacterium tumefaciens st. 4404 was electroporated under several conditions in the presence of pBI121 plasmid (which also confers kanamycin resistance and Gus activity). The electroporated bacteria have been plated on kanamycin-containing medium in order to select transformants (11).

C. Plans: Continue to process Group 29 tobacco roots into ammonium sulfate fraction, and continue to prepare PMT samples for the program. Continue studies on PMT renaturation. Continue evaluation of PMT enzyme assay protocol. Continue work with HPLC to resolve 60kD protein. Continue cultures of electroporated Burley 21 cells. Continue to obtain amino acid sequence of fragments of the putative PMT enzyme. Continue to accumulate DNA sequence data for tobacco root overly expressed clones. Continue to sequence PCR clone.

D. References:

1. Lyle, J. Notebook No. 9077, p. 34.

2. Davies, S. Notebook No. 9103, p. 58.

3. Turner, D. Notebook No. 9094, p. 112.

4. Steele, M. Notebook No. 9068, p. 44.

5. Yu, T. Notebook No. 9002, p. 139.

6. Nakatani, H. Notebook No. 8384, p. 189.

7. Bower, P. Notebook No. 9032, pp. 76-91.

8. Wahab, S. Notebook No. 8130, pp. 81-82.

9. Malik, V. Notebook No. 8974, p. 122.

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10. Michalik, T. Notebook No. 9109, p. 79.

11. Vaughan, B. Notebook No. 8948, p. 141.

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